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Maternal control of axial–paraxial mesoderm patterning via direct transcriptional repression in zebrafish

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ABSTRACT

Axial–paraxial mesoderm patterning is a special dorsal–ventral patterning event of establishing the vertebrate body plan. Though dorsal–ventral patterning has been extensively studied, the initiation of axial–paraxial mesoderm pattering remains largely unrevealed. In zebrafish, *spt* cell-autonomously regulates paraxial mesoderm specification and *flh* represses *spt* expression to promote axial mesoderm fate, but the expression domains of *spt* and *flh* initially overlap in the entire marginal zone of the embryo. Defining *spt* and *flh* territories is therefore a premise of axial–paraxial mesoderm patterning. In this study, we investigated why and how the initial expression of *flh* becomes repressed in the ventrolateral marginal cells during blastula stage. Loss- and gain-of-function experiments showed that a maternal transcription factor Vsx1 is essential for restricting *flh* expression within the dorsal margin and preserving *spt* expression and paraxial mesoderm specification in the ventrolateral margin of embryo. Chromatin immunoprecipitation and electrophoretic mobility shift assays in combination with core consensus sequence mutation analysis further revealed that Vsx1 can directly repress *flh* by binding to the proximal promoter at a specific site. Inhibiting maternal *vsx1* translation resulted in confusion of axial and paraxial mesoderm markers expression and axial–paraxial mesoderm patterning. These results demonstrated that direct transcriptional repression of the decisive axial mesoderm gene by maternal ventralizing factor is a crucial regulatory mechanism of initiating axial–paraxial mesoderm patterning in vertebrates.

Introduction

Dorsal–ventral (DV) patterning is an early development program of establishing the animal body plan. Axial–paraxial mesoderm patterning, by which the domains for generation of the notochord and flanking mesoderm are defined, is a vertebrate-specific DV patterning event. It has been confirmed that, after the establishment of DV polarity, the mesoderm is induced by Nodal signaling with low level at the ventral side and high level at the dorsal side (Green and Smith, 1990; Green et al., 1992; Gurdon et al., 1994; McDowell and Gurdon, 1999; Gurdon and Bourillot, 2001; Shen, 2007). But there is no compelling evidence supporting that a reduction of Nodal signaling in the dorsal side results in a respecification of dorsal to ventral fates (Kimelman, 2006). Experiment in zebrafish provides evidence that DV patterning of mesoderm is independent of Nodal signals (Dougan et al., 2003). A zygotic bone morphogenetic protein (Bmp) activity gradient, generated by antagonistic actions between Bmps and BMP antagonists emanated from the dorsal organizer, plays an important role in defining distinct ventrolateral fate domains along the DV axis during gastrulation (Dosch et al., 1997; Graff, 1997; Jones and Smith, 1998; Nguyen et al., 1998; Barth et al., 1999; Dale and Wardle, 1999; De Robertis et al., 2000). However, axial mesoderm is largely unaffected in Bmp pathway mutants, implicating that the zygotic Bmp activity gradient is not involved in defining the axial and paraxial mesoderm domains. In zebrafish, ventrally expressed zygotic Wnt8 activates *vox*/*vent*/*ved* gene family in cooperation with Bmp2b to repress dorsal gene expression and maintain ventrolateral identity during gastrulation (Melby et al., 2000; Imai et al., 2001; Lekven et al., 2001; Shimizu et al., 2002; Ramel and Lekven, 2004; Ramel et al., 2005). But the initial distinction between axial and non-axial domains at 30–40% epiboly was unaffected in the embryos lacking the functions of both zygotic Wnt8 and Bmp2b signaling pathways (Reim and Brand, 2006; Ramel et al., 2005). Recent study further demonstrated that zebrafish maternal Wnt8 is located at the dorsal side after fertilization and functions as a dorsal determinant during blastula stage (Lu et al., 2011). Therefore, Wnt8 and Bmp2b signaling

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pathways are unlikely involved in initiating ventral fate before gastrulation. The ventral specification of mesoderm is actively regulated by maternal ventralizing factor via suppressing maternal dorsalizing signals (Itoh and Sokol, 1999; Kuhl et al., 2000; Saneyoshi et al., 2002) or activating zygotic ventral genes, such as *bmps* and *vox*/*vent*/*ved* gene family (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003; Reim and Brand, 2006; Flores et al., 2008). Among the three maternal ventralizing factors identified in zebrafish, maternal Radar and Pou2 do not influence the expression domain of dorsal organizer genes (Sidi et al., 2003; Reim and Brand, 2006), suggesting that these maternal factors are unlikely involved in defining axial and paraxial mesoderm domains. Maternal Runx2bt2 activates *vent*, *vox* and *ved* to promote non-axial mesoderm fate and can influence the distinction between axial and paraxial mesoderm domains at 50% epiboly stage (Flores et al., 2008). However, it remains unclear whether this indirect regulation is involved in initiating or maintaining the distinction between axial and paraxial mesoderm domains. Taken together, the initial regulation of axial–paraxial mesoderm patterning remains unclear.

The regulation of axial and paraxial mesoderm specification has been intensively studied in zebrafish. A transcriptional factor Spadetail (Spt) cell-autonomously regulates paraxial mesoderm specification in the ventrolateral margin of early embryo (Kimmel et al., 1989; Ho and Kane, 1990; Griffin et al., 1998; Amacher and Kimmel, 1998), and loss of Spt function can elicit the ventral expansion of the axial mesoderm domain and the absence of paraxial mesoderm marker expression (Thisse et al., 1995; Hammerschmidt et al., 1996). A homeodomain transcriptional factor Floating head (Flh) represses *spt* expression to promote axial mesoderm fate in the dorsal margin (Amacher and Kimmel, 1998; Yamamoto et al., 1998). In *flh* mutant embryos the axial mesoderm cell-autonomously converts into paraxial mesoderm (Talbot et al., 1995; Halpern et al., 1995; Melby et al., 1996; Amacher and Kimmel, 1998), although dispersed *flh* mutant cells can differentiate into notochord cells in response to notochord-promoting signals in the wild-type host embryo (Amacher and Kimmel, 1998). Interestingly, the expression domains of *spt* and *flh* initially overlap in the entire margin zone of the embryo at dome stage and are divided from 30% epiboly stage (Griffin et al., 1998; Talbot et al., 1995). Therefore, rapid repression of *flh* in the ventrolateral marginal cells from dome stage to 30% epiboly stage is essential for maintaining *spt* expression in the ventrolateral margin and a premise of axial and paraxial mesoderm patterning. Investigating how the initial expression of *flh* in the ventrolateral margin is inhibited during late blastula stage will gain an insight into the initial axial–paraxial mesoderm patterning.

A paired-like transcription factor gene *visual system homeobox-1* (*vsx1*) which encodes a protein containing homeodomain and CVC domain has been cloned in several vertebrate species (Levine and Schechter, 1993; Levine et al., 1994; Passini et al., 1998; Semina et al., 2000; Ohtoshi et al., 2001; D'Autilia et al., 2006). Vsx1 plays an important role in regulating retinal progenitor cells proliferation and differentiation, and in maintaining the function of bipolar cells in vertebrates (Héon et al., 2002; Ohtoshi et al., 2004; Valleix et al., 2006; Clark et al., 2008). Since *vsx1* transcripts were detected in zebrafish maternal mRNA pool and at early developmental stage in all the examined vertebrate species (Levine and Schechter, 1993; Levine et al., 1994; Passini et al., 1998; Semina et al., 2000; Ohtoshi et al., 2001; D'Autilia et al., 2006), it has been reasonably postulated that *vsx1* might play an important role during early embryogenesis (Ohtoshi et al., 2001). Here, we show that Vsx1 protein encoded by maternal *vsx1* mRNA can directly repress *flh* transcription to preserve *spt* expression and paraxial mesoderm specification in the ventrolateral margin of blastula embryo. In this way, the original overlapped axial and paraxial mesoderm domains are divided and the initial distinction between axial and paraxial mesoderm domains takes shape.

Results

Maternal Vsx1 is essential for normal paraxial mesoderm specification and axial–paraxial mesoderm patterning

To determine whether *vsx1* has a role in regulating early embryogenesis in zebrafish, endogenous *vsx1* was knocked down by injecting translation blocking MO (tbMO) at one cell stage. When 8 ng tbMO was injected, 89.4% of embryos ($N=152$) were arrested at the onset of gastrulation and died soon. When the dose was reduced to 4 ng, the percentage of dead embryos was reduced to 14% ($N=164$), 58% of the embryos at 24 hours post-fertilization (hpf) exhibited a morphant specific phenotype with no obvious paraxial somites but disorganized dorsal midline structures (Fig. 1D–F). When the dose was reduced to 2 ng per embryo, the ratio of abnormal embryos was very low and the abnormalities varied. Therefore, 4 ng of *vsx1* tbMO was used for subsequent experiments. The specificity of *vsx1* tbMO was verified in rescue experiment by co-injecting with *vsx1* mis-mRNA (encoding the normal Vsx1 protein but lacking the target site of the *vsx1* tbMO). Coinjection of *vsx1* mis-mRNA elicited conversion of the morphant phenotype into normal or *vsx1* overexpression phenotypes (described below) and decrease of lethality in a dose-dependent manner (Fig. 1M).

To understand how Vsx1 regulates the dorsal structure development, we examined the axial and paraxial mesoderm formation by visualizing the expression domains of an axial mesoderm marker gene *ntl* and a paraxial mesoderm marker gene *myoD*, respectively, in *vsx1* knockdown embryos at bud stage and 8–10 somite stage. Whole mount in situ hybridization showed that the formation of both axial and paraxial mesoderm was abnormal in *vsx1* tbMO injected embryos. In comparison with wild type, the *ntl* marked axial mesoderm domain in the *vsx1* knockdown embryos was expanded in width but shortened in length (Fig. 2C–F and Fig. S1E and F), while *myoD* marked paraxial mesoderm domain in the *vsx1* knockdown embryos was suppressed in various degrees with defects of convergence and somitogenesis (Fig. 2M–P and Fig. S1G and H). The suppression of paraxial mesoderm formation in *vsx1* morphants was confirmed by examining the expression of two other paraxial mesoderm marker genes *msgn1* (Yoo et al., 2003; Fior et al., 2012) and *tbx24* (Nikaido et al., 2002) at middle gastrula stage. When 4 ng *vsx1* tbMO was injected at one cell stage, the expression of *msgn1* at the ventrolateral region was significantly repressed in 61% of the embryos ($N=36$, Fig. 3, A–F), and *tbx24* at the paraxial region was significantly repressed in 69% of the embryos ($N=46$, Fig. 3G–L). These results suggest that Vsx1 is essential for promoting normal paraxial mesoderm specification and axial–paraxial mesoderm patterning during early embryogenesis.

We further examined the function of *vsx1* in regulating early embryogenesis by overexpression. When 200 pg *vsx1* mRNA was injected at one cell stage, 57.7% of the embryos ($N=156$) exhibited widely bifurcated paraxial mesoderm domains with no distinguishable dorsal midline structures in the dorsal center region at 24 hpf (Fig. 1G–I). Expression analysis of axial and paraxial mesoderm marker genes showed that, at the anterior axial region, *ntl* marked axial mesoderm specification was severely suppressed (Fig. 2G–J and Fig. S1I and J), while dispersed *myoD* marked paraxial mesoderm cells were detectable (Fig. 2Q–T). *myoD* marked paraxial mesoderm and somites were formed at the ventrolateral region but failed in converging to the normal dorsal position (Fig. 2Q–T and Fig. S1K and L). These results suggest that *vsx1* is able to repress axial mesoderm specification at early developmental stage.